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**EXPRESSION OF MOLECULAR MARKERS IN
BRAIN, SERUM, AND LUNG TISSUES FOLLOWING
HYPOBARIC HYPOXIA**

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14. In the last decade, hypoxia has come to the forefront as a possible contributing factor to the physiological incidents experienced by pilots of high performance aircraft. Physiological incidents have been reported by pilots of F-22, T-45, F-35 and T-6 aircraft. Due to the lack of in-flight monitoring, post-flight monitoring of biological markers is one way of determining the nature of these incidents. In this study, Sprague Dawley rats were exposed to atmospheric pressures equivalent to 22,500 feet altitude and oxygen concentrations of either 21% or 94% for four hours. These conditions mimic the most oxygen-deficient conditions and the ideal conditions potentially experienced by pilots at the highest cockpit altitude of an F-22 aircraft, respectively. At different times after exposure (0, 1, 3 and 24 hours), blood, brain, and lung tissues were collected. These tissues were tested for levels of hypoxic markers, hypoxia-inducible factor-1 α (HIF-1 α), prolyl-4-hydroxylase (PHD), vascular endothelial growth factor (VEGF), and erythropoietin (EPO), to determine post-incident exposure profiles. The most prominent increases were observed in lung and serum EPO levels at 0, 1, and 3 hours after exposure. Serum EPO could be a useful indicator of hypoxic exposure in pilots.					
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PREFACE

Funding for this project was provided through Aerospace Toxicology, which is part of the Aerospace Physiology and Toxicology Program in the 711th Human Performance Wing of the Air Force Research Laboratory. This research was conducted under cooperative agreement FA8650-15-2-6608 with the Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF). The program manager for the HJF cooperative agreement was David R. Mattie, PhD (711 HPW/RHDJ).

The protocol “Identification and Optimization of Markers of Hypobaric Hypoxia in Sprague Dawley Rats (*Rattus norvegicus*)” was approved by the Wright-Patterson AFB Institutional Animal Care and Use Committee (IACUC) as protocol number F-WA-2016-0163. The study was conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), International, in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011). The study was performed in compliance with DODI 3216.1.

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1.0 SUMMARY

In the last decade, hypoxia has come to the forefront as a possible contributing factor to the physiological incidents experienced by pilots of high performance aircraft. These incidents were responsible for stopping F-22 flights in 2011 and may have contributed to minor deficits in brain function of pilots. During the summer of 2017, T-45 training jets were subject to flight stoppage and altitude restrictions pending investigation of physiological incidents, and F-35's at one base were briefly grounded. Since then, similar physiological events have also been reported in F/A-18 and T-6 aircraft. Due to the lack of in-flight monitoring of breathing gases, post-flight monitoring of biological markers is one way of determining the nature of in-flight incidents, after the fact, both in animal models and in real-world pilots. The purpose of this study was to determine the time course of expression of hypoxic markers following a hypobaric hypoxia incident. This study is based on an animal model exposed to hypobaric hypoxia, mimicking conditions experienced in fighter jet cockpits. Levels of specific hypoxic markers in the blood and tissues were examined as indicators of whether or not hypoxic conditions had occurred. The goal of this study was to identify the best markers of hypobaric hypoxia in the rodent model, in which tissues they appear and when they can be detected after exposure.

In this study, Sprague Dawley rats were exposed to atmospheric pressures equivalent to 22,500 feet altitude and oxygen concentrations of either 21% or 94% for four hours. These conditions mimic the most oxygen-deficient conditions and the relevant operational conditions potentially experienced by pilots at the highest cockpit altitude of an F-22 aircraft. At sea level, 21% oxygen is the norm, but at 22,500 feet above sea level, oxygen concentrations below 50% do not provide enough to maintain normal oxygen levels in the blood, leading to hypoxia. At different times after exposure (0, 1, 3 and 24 hours), blood, brain, and lung tissues were collected. These tissues were tested for levels of hypoxic markers, hypoxia-inducible factor-1 α (HIF-1 α), prolyl-4-hydroxylase 2 (PHD2), vascular endothelial growth factor (VEGF), and erythropoietin (EPO), to determine post-incident exposure profiles.

HIF-1 α levels in brain stem, cerebellum and cortex did not differ among control (21% O₂, 1,000 ft above sea level), hypoxic (21% O₂, 22,500 ft above sea level) and pilot (94% O₂, 22,500 ft above sea level) exposures. PHD2 levels appeared to be lower in brain stem and cerebellum immediately following hypoxic exposure, increasing to near control levels by 24 hours post-exposure. VEGF levels increased in brain stem and decreased in lung following hypoxic exposure. Lung and serum EPO levels were significantly increased at 0, 1, and 3 hours following hypoxic exposure. By 24 hours post-exposure, the markers examined in this study had returned to control levels, with the exception of VEGF in hypoxia-exposed brain stem.

2.0 INTRODUCTION

An increase in hypoxia-like incidents experienced by pilots of high performance aircraft during the last several years has brought hypoxia to the forefront in aerospace research. These incidents were responsible for grounding the F-22 fleet in 2011 and have been implicated in catastrophic loss of life and aircraft as well as temporary, minor deficits in pilot cognitive performance (summarized in West, 2013). More recently, significant increases in the occurrence of physiological incidents among Air Force, Navy, and Marine pilots of F/A-18 Hornet, F-35A, T-6, and T-45 aircraft have re-focused attention on pilot oxygen availability. While a host of sensors is employed to monitor the status of an aircraft in-flight, there are no sensors that monitor the oxygen status of the pilot. In the absence of real-time sensors of pilot conditions and physiology, we must be able to detect specific markers of hypoxia after an incident has occurred in order to determine the nature of the incident. Results of post-exposure monitoring will enable researchers or flight doctors to determine whether or not real-world incidents are due to hypoxia.

The human and the aircraft OBOGS (on-board oxygen generating system) are the two elements potentially contributing to the onset of unexplained physiological episodes. Pilots of high-performance fighter jets are subject to a wide range of environmental stressors during flight. They experience rapid acceleration up to 9G, hypobaric pressures to the equivalent of 22,500 feet above sea level, thermal stressors, and the mental stressors associated with mission performance and aircraft operation. In order to avoid G-LOC, or G-related loss of consciousness, pilots wear full anti-gravity suits. Pressurized vests are worn to prevent overinflation of the lungs (West, 2013). Additionally, pilots operate in a tightly confined space. The OBOGS is a critical life-support system, responsible for delivering breathing air to the pilot. This system operates using conditioned engine bleed air, concentrates the oxygen, removes nitrogen, and provides an enriched level of oxygen to the pilot through a demand valve in the breathing apparatus. Each airframe has its own OBOGS with slightly different operational parameters. The maximum O₂ concentration attainable by the F-22 OBOGS is 94%.

The system in question, which includes both the pilot and the aircraft, is highly complex. Real-time monitoring of pilot physiology could enable rapid identification of the cause of any physiological incidents. However, in the absence of such monitoring, post-flight measurement of physiological parameters may provide valuable insight into the root cause of in-flight incidents. This study was undertaken in order to characterize the response to hypobaric hypoxia in an animal model, with the expectation that the results may eventually be applied to post-flight monitoring of pilots through combined modeling efforts and knowledge gained in human studies.

When subjected to hypoxic conditions, the body responds by increasing respiration and heart rate. These rapid physiological responses ensure that the brain continues to receive sufficient oxygen to carry out normal processes. However, when oxygen levels are depleted to the point where increased respiration and cardiac output cannot supply enough oxygen to the brain, cellular hypoxic responses begin to occur. Transcription factor HIF-1 is the initiator of these cellular responses.

Under normoxic conditions, HIF-1 α is undetectable within the cell, because it is rapidly hydroxylated by prolyl-4-hydroxylase (PHD), ubiquitinated, and degraded. When hypoxic

conditions are encountered, HIF-1 α builds up in the intracellular space and dimerizes with HIF-1 β . The HIF-1 heterodimer then translocates to the nucleus, where it modifies gene transcription by binding to hypoxia-responsive elements. Downstream effects of this modified gene transcription include glycolysis, apoptosis, erythropoiesis (EPO), vascular remodeling (VEGF) and cell cycle arrest (Semenza, 2004).

This study employed a rat model of hypobaric hypoxia, mimicking the maximum cockpit altitude and the lowest and highest potential oxygen concentrations (21% and 94%, respectively) experienced by F-22 pilots, to examine a battery of putative hypoxic markers. The goal of the study was to induce hypobaric hypoxia and identify specific markers of the condition, in which tissues they appear and at what time(s) they can be detected after exposure. This study examined a range of hypoxic markers found in the literature, including hypoxia-inducible factor-1 α (HIF-1 α ; Wang *et al.*, 1995; Semenza, 1999; Semenza, 2004), prolyl-4-hydroxylase (PHD; Berra *et al.*, 2003; Metzen *et al.*, 2005), vascular endothelial growth factor (VEGF; Levy *et al.*, 1995; Hoeben *et al.*, 2004) and erythropoietin (EPO; Abbrecht and Littell, 1972; Eckhardt *et al.*, 1988; Cahan *et al.*, 1990; Ge *et al.*, 2002).

The markers selected for analysis in this study each play a unique role in the hypoxic response. Most notably, HIF-1 α is the transcription factor that initiates the response to decreased oxygen in cells. HIF-1 α is responsible for regulating erythropoiesis, angiogenesis and glycolysis, among other things, and has been linked to transcriptional regulation of over 70 genes (Semenza, 2004). HIF-1 α is rapidly degraded under normoxic conditions, but when hypoxia occurs, it accumulates and dimerizes with a HIF- β subunit, translocates to the nucleus and begins affecting transcription of hypoxia responsive genes. The half-life of HIF-1 α is very brief. Studies have determined that HIF-1 α lasts less than five min. in tissue culture, and less than one min. in isolated perfused lung tissue upon restoration of normoxia (Wang *et al.*, 1995; Yu *et al.*, 1998).

Prolyl-4-hydroxylase 2 (PHD2) is one of a family of enzymes, including two other isoforms (PHD1 and PHD3) which play a role in collagen formation. PHD2 also plays a critical role in regulating the hypoxic response by hydroxylation of HIF-1 α under normoxic conditions. PHD's are made up of four subunits: two α , and two β . The α subunit consists, in part, of the P4Ha1 peptide, which contains the catalytically active site of the enzyme. P4Ha1 was targeted for analysis in this study due to the availability of an ELISA detection kit. For the purpose of this study, we have assumed that P4Ha1 levels directly reflect PHD2 levels. Under conditions with sufficient cellular oxygen, hydroxylation of HIF-1 α by PHD2 leads to ubiquitination and degradation, preventing activation of the hypoxic response. One study of HeLa and CAL51 cell lines indicated that PHD2 expression is transcriptionally elevated during a hypoxic insult (Berra *et al.*, 2003). Another study found that the PHD2 gene is directly regulated by a hypoxia-responsive element (Metzen *et al.*, 2005). In combination, these results indicate that PHD2 expression and activity are tightly regulated via a feedback mechanism.

VEGF plays a critical role in angiogenesis during normal physiological processes and also disease processes, such as cancer tumorigenesis (reviewed by Hoeben *et al.*, 2004). Hypoxic conditions trigger local or regional angiogenesis in order to deliver oxygen to tissues in need. VEGF gene transcription is directly regulated by a hypoxia-responsive element (Semenza, 1999) and many previous studies have shown increased VEGF expression following hypoxia (Ferrara,

1999). For example, Levy and colleagues found that, in PC12 cells, hypoxia increased VEGF mRNA levels 12-fold while protein levels increased approximately 3-fold (Levy *et al.*, 1995). In a study of male Wistar rats, brain microvasculature increased significantly after 1, 2, and 3 weeks of hypoxia, but did not increase after 4 days of hypoxia. This study also showed that three weeks of normoxic recovery lead to decreased microvasculature down to pre-hypoxic levels (Harik *et al.*, 1996). Increases in both VEGF transcription and mRNA stability contribute to higher levels of VEGF (Ikeda *et al.*, 1995; Levy *et al.*, 1996) and changes in VEGF receptor density have also been noted in hypoxia (reviewed in Ferrara, 1999).

Erythropoietin (EPO) is a hormone responsible for regulating the oxygen-carrying capacity of the blood. It is produced primarily by the kidney and acts in the bone marrow, where it leads to the development of mature red blood cells, thereby increasing the oxygen-carrying capacity of the blood (Schuster *et al.*, 1987). EPO receptors are also found in brain tissue of rodents and primates, and EPO seems to play a role in protecting brain tissue from excitotoxicity and ischemic damage (Siren *et al.*, 2000; Morishita *et al.*, 1997; Sadamoto *et al.*, 1998; Sakanaka *et al.*, 1998). Many studies have shown an increase in serum EPO levels as a result of hypoxic and hypobaric-hypoxic exposures. In a study by Schuster *et al.*, (1987), rats exposed to hypobaric hypoxia for four hours had elevated EPO in the kidney within the first hour, with peak levels at the end of the exposure. Serum EPO levels followed a similar profile that was slightly delayed, with the highest expression occurring one hour after the end of exposure and then gradually declining during the following two hours (Schuster *et al.*, 1987). A study by Cahan and others found that EPO in rats was elevated as early as 30 minutes after the onset of hypobaric hypoxia exposure (1990) and Eckhardt *et al.* (1988) found that human EPO elevation occurred as early as 84 minutes after the onset of exposure to hypobaric hypoxia (at 13,120 feet above sea level). Within one hour of exposure to 22,000 feet altitude, Schooley and Mahlmann found that serum EPO was elevated. EPO level peaked two hours after the exposure ended and continued to decline over the next 18 hours (Schooley and Mahlmann, 1972). Each of these results seem to support the observation that the higher the altitude, the larger the increase in serum EPO (Abbrecht and Littell, 1972; Ge *et al.*, 2002).

In this study, male Sprague Dawley rats were exposed to atmospheric pressures equivalent to 22,500 feet altitude and oxygen concentrations of 21% or 94% for four hours, plus ten minutes each to ascend and descend. These conditions mimic the lowest and highest potential oxygen exposures at the maximum cockpit altitude in an F-22 aircraft. At 0, 1, 3, or 24 hours after the exposure, rats were euthanized via an American Veterinary Medical Association (AVMA) approved method. Blood, brain, and lung tissues were collected because of their key roles in responding to hypoxia. Cerebral cortex, hippocampus, brain stem and cerebellum were dissected and used for downstream biochemical assays. Tissues were analyzed for levels of HIF-1 α , P4Ha1, VEGF, and EPO. Finally, some data were compared against previously published results as part of our hypoxia modeling effort.

This study examined the molecular responses in brain (hippocampus, brain stem, cerebral cortex and cerebellum), serum, and lung to hypoxic-hypobaric conditions (21% O₂ at 22,500 ft above sea level) and to pilot conditions (94% O₂ at 22,500 ft above sea level) in order to determine both the degree of hypoxia achieved in these tissues, and to assess the sensitivity and post-exposure profile of each marker.

3.0 METHODS

3.1 Altitude Chamber

The altitude system consists of a custom built 20"x20.5"x20" stainless steel chamber (Laco Technologies, Salt Lake City, UT) with 27 ports and a 1.5" thick acrylic door (Figure 1). The chamber can withstand hypobaric conditions to an equivalent altitude of 130,000 feet. The chamber pressure was monitored from three distributed ports using oxygen cleaned, intrinsically safe, pressure sensors from American Sensors Technology (AST, Mt Olive, NJ). Three whole body exposure chambers were fitted within the altitude chamber to contain two rats each, and the test atmosphere. The test atmosphere was generated using Teledyne mass flow controllers (Teledyne-Hastings, Hampton, VA), supplied from certified compressed gas cylinders, and transported within stainless steel tubing with an inert coating. Immediately upon exiting the whole body chamber, test atmospheres consisting of greater than 21% O₂ were mixed with nitrogen within the system such that the vented atmosphere contained no more than 20% O₂. The simulated altitude was achieved and maintained by an Edwards 10iC vacuum pump with an inline Aalborg needle valve (Edwards Vacuum, Glenwillow, OH). Test atmosphere and simulated altitude were controlled via a computer interface with customizable programs created in LabVIEW (Austin, TX). For the purposes of this report the term altitude refers to the equivalent pressure. The simulated altitude (1,000 ft) was also maintained for the control group to mimic all conditions of exposure, although an adjustment of less than 100 ft was needed as the laboratory is over 900 ft above sea level. For ease of observation, the altitude chamber is fitted with a ceiling-mounted camera, hard-wired to a monitor for live viewing of animals throughout exposures.

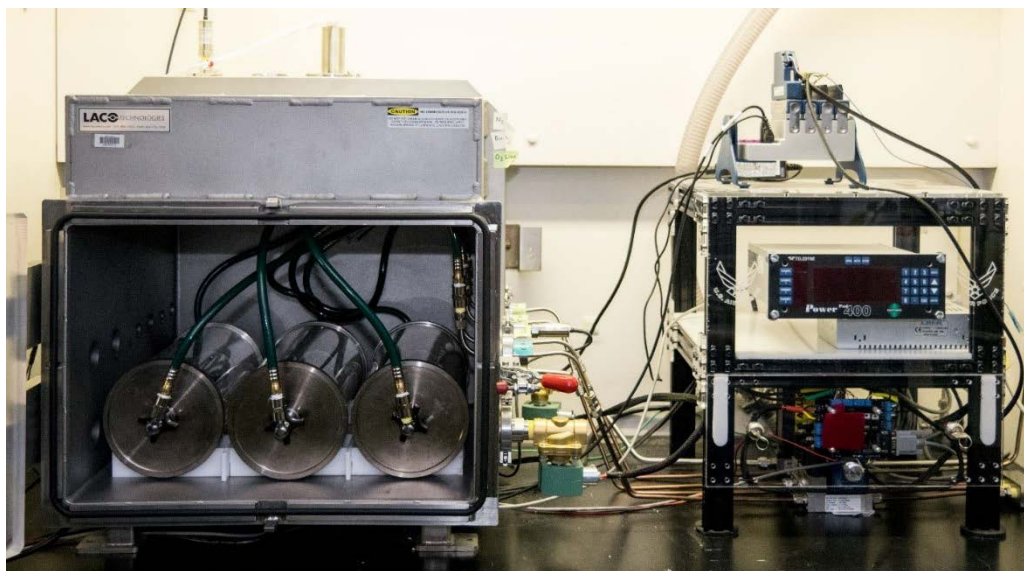


Figure 1. Altitude chamber (Left) and Control Tower (Right). Stainless steel altitude chamber with three interior, individually plumbed, animal chambers and Teledyne mass flow controller with stainless steel tubing for transport of gases to altitude chamber.

3.2 Animal Handling/Housing

3.2.1 Acclimation to Reverse Light/Dark Cycle

Upon arrival at the facility, all animals were subjected to a two-week acclimation period that reversed the light/dark cycle (lights on from 1800 to 0600), and ran concurrently with the quarantine period. The reversed light/dark cycle was imposed to ensure that the animals would be maximally active during the exposures to altitude and hypoxia and that their oxygen consumption would better reflect that of an actively engaged pilot. All health checks, acclimations to interior holding chambers, and exposures took place under red lights. Animal cages were covered to maintain darkness during transport to and from housing and exposure rooms.

3.3 Exposure Scenario

Two male Sprague Dawley rats weighing approximately 200 g each were acclimated to each interior holding chamber on three separate days for 1 hr a day. Acclimations were completed no more than one week before exposures. The three exposure groups were: 1. Control (CTL) – pressure equivalent to 1,000 ft above sea level and 21% O₂; 2. Pilot Control (PIL) – pressure equivalent to 22,500 ft above sea level and 94% O₂; 3. Hypoxia (HYP) – pressure equivalent to 22,500 ft and 21 % O₂. Six rats were exposed at a time. All CTL animals were necropsied directly after being removed from the exposure chamber. For PIL and HYP exposure groups, an n of six animals was collected at each of four time points (0, 1, 3 and 24 hours post-exposure). For exposures, two acclimated rats were placed in each of the three interior holding chambers, and the lids were placed and clamped shut. The altitude chamber door was shut and locked. The appropriate program in LabVIEW was initiated for the desired exposure. For altitude exposures, the LabVIEW program was set to increase the altitude 2,250 ft/min. After the 10 min ascent, the altitude was maintained at 22,500 ft for 4 hr. After 4 hr the altitude was decreased by 2,250 ft/min. Total exposure time for CTL, PIL and HYP groups was 4 hr and 20 min. Time 0 rats were euthanized by rapid decapitation within 10 min of the end of the exposure, others were euthanized at 1 hr, 3 hr, or 24 hr after exposure. Trunk blood was collected in 15 mL Falcon tubes and allowed to clot on ice. Tubes were centrifuged at 2,500xg for 10 min at 4°C, and serum was collected. Brains were removed and dissected into cortex, cerebellum, hippocampus and brain stem. Heart and lung tissues were also removed. All tissues were flash frozen in liquid nitrogen and stored at -80°C. All brain regions and lung tissues were subsequently ground in liquid nitrogen in order to provide homogenous sample portions for all assays. Hippocampus was the smallest brain region collected, and because of limited tissue weight was assayed only for the presence of P4Ha1. Heart tissues remained unground at -80°C. Heart tissue was not analyzed for this study.

3.4 Collection of Body Weight Measurements

Animal weights were collected (using a Sartorius Entris 2202-1S scale) and recorded before each exposure, and just before necropsy for the 1-, 3-, and 24-hour time points. Due to time constraints, the time 0 animals and CTL animals were not weighed prior to necropsy.

3.5 Analysis of Hypoxic Indicators

Approximately 60 mg of each of the ground tissues were weighed into cold tubes and lysed in 1,000 μ L of NP-40 cell lysis buffer (Invitrogen, Camarillo, CA) containing protease inhibitor cocktail (Sigma, St. Louis, MO). Briefly, lysis buffer was added to the tube of ground tissue (on ice) and samples were sonicated at amplitude 30 for 30 sec using a probe sonicator (Fisher Scientific, Pittsburgh, PA). Sonicated samples were incubated for two hours at 4°C with constant agitation, then centrifuged for 20 min at 12,000xg, 4°C in a microcentrifuge. Supernatant was retained and pellet discarded. Each sample lysate was analyzed in duplicate using the bicinchonic acid assay (Pierce™ BCA Protein Assay Kit; Pierce Biotechnology, Rockford, IL) according to manufacturer's protocol to determine the total protein concentration. All data were analyzed using Prism® 5 software (GraphPad, La Jolla, CA).

3.5.1 Hypoxia Inducible Factor-1 α Enzyme-Linked Immunosorbent Assay (ELISA)

Brain stem, cerebellum, cerebral cortex and lung tissue lysates from time 0 animals were analyzed by ELISA for the presence of both phosphorylated and unphosphorylated HIF-1 α (DuoSet® IC Human/Mouse Total HIF-1 α ELISA; R&D Systems, Minneapolis, MN). Samples were analyzed in duplicate with no dilution. Assay was performed according to manufacturer's protocol, with the exception of the standard curve being shifted lower to avoid exceeding the upper detection limit of the spectrophotometer (Molecular Devices, Sunnyvale, CA).

3.5.2 Prolyl-4-hydroxylase Alpha Polypeptide I ELISA

Brain stem, hippocampus, cerebellum and cerebral cortex tissue lysates from all animals were analyzed by ELISA for the presence of prolyl-4-hydroxylase alpha polypeptide I (P4Ha1; Biomatik, Wilmington, DE). P4Ha1 is a subunit of Prolyl-4-hydroxylase, the enzyme responsible for hydroxylating, and thereby deactivating, HIF-1 α . A preliminary ELISA revealed no detection of P4Ha1 in lung tissue from CTL, PIL, or HYP exposures, so this tissue was not included in the assay. Samples were analyzed in duplicate with no dilution. Assay was performed according to manufacturer's protocol.

3.5.3 Vascular Endothelial Growth Factor ELISA

Brain stem, cerebellum, cerebral cortex, and lung tissue lysates from all animals were analyzed by ELISA (R&D Systems, Minneapolis, MN) for the presence of VEGF. Brain tissue lysates were analyzed undiluted and lung tissue lysates were diluted 1:2 in reagent diluent in order to obtain results within the range of the standard curve. The assay was performed as described in the manufacturer's protocol.

3.5.4 Erythropoietin ELISA

Serum and brain stem and lung tissue lysates were analyzed by ELISA (BioLegend, San Diego, CA) for the presence of erythropoietin. Lung and brain stem lysates were analyzed undiluted. CTL and PIL sera were undiluted, while HYP sera were diluted between 1:2 and 1:25 in order to obtain readings within the range of the standard curve. Cerebral cortex and cerebellum were not

assayed for the presence of EPO because they were found to have undetectable levels of EPO during a preliminary ELISA.

3.6 Modeling and Simulation of Hypoxic Indicators

Elsewhere we have developed a mathematical model for the HIF-1 α signaling pathway and associated downstream events (Robinson *et al.*, 2017), in terms of a detailed time-course model of HIF-1 α responses to ambient oxygen levels. This model also enabled us to relate steady-state (SS) gene transcription products to ambient oxygen concentrations (in a “dose/response” manner), without fully parameterizing the kinetic model. This model did not directly incorporate gene transcription; however, once transported into the nucleus of the cell, HIF-1 α was assumed to bind with hypoxia responsive element (HRE) on the DNA with a bimolecular rate constant, and be degraded according to a first order rate constant. The binding of HIF-1 α to HRE initiates a transcription signal, which is assumed to mediate gene transcription. The model was thus able to provide an estimate of dose-response, where the “dose” is the ambient oxygen level, and the “response” is the transcription signal. Assuming that further downstream effects (such as VEGF production or message mRNA) are linearly related to this transcription signal allows the model to be applied to, for example, VEGF changes (relative to control) due to altered ambient oxygen levels (relative to normal oxygen). This approach was initially applied to the data of Schoch *et al.* (2002) (Robinson *et al.*, 2017), and can also be applied to some of the data presented here.

4.0 RESULTS

4.1 Body Weight Changes

Significant differences in body weight change were observed between PIL and HYP animals at each of the time points collected (1, 3, and 24 hours; ***p<0.001; ****p<0.0001; **p<0.01, respectively; Fig. 2). PIL animal weights were consistently increased over pre-exposure weights at all times measured, whereas HYP animal weights were decreased as much as 2.5% at 1 hour after exposure. By 24 hours after exposure, PIL weights were 3.6% higher than pre-exposure weight, while HYP animal weights were only 1.6% above pre-exposure values (see Table 1.)

Table 1. Average Percent Body Weight Change in Pilot- and Hypoxia-Exposed Groups

Group	1 Hr		3 Hrs		24 Hrs	
	Pre-Exposure Weight	% Weight Change	Pre-Exposure Weight	% Weight Change	Pre-Exposure Weight	% Weight Change
Control	331.2					
Pilot	328.2	0.2	339.3	1.6	335.7	3.6
Hypoxic	329.5	-2.5	320.3	-1.5	316.8	1.6

Notes: Group average animal weight before exposures and average percent change in body weight at necropsy at times 1, 3, and 24 hours post-exposure. Control group were necropsied immediately following exposure therefore only one average pre-exposure weight is reported.

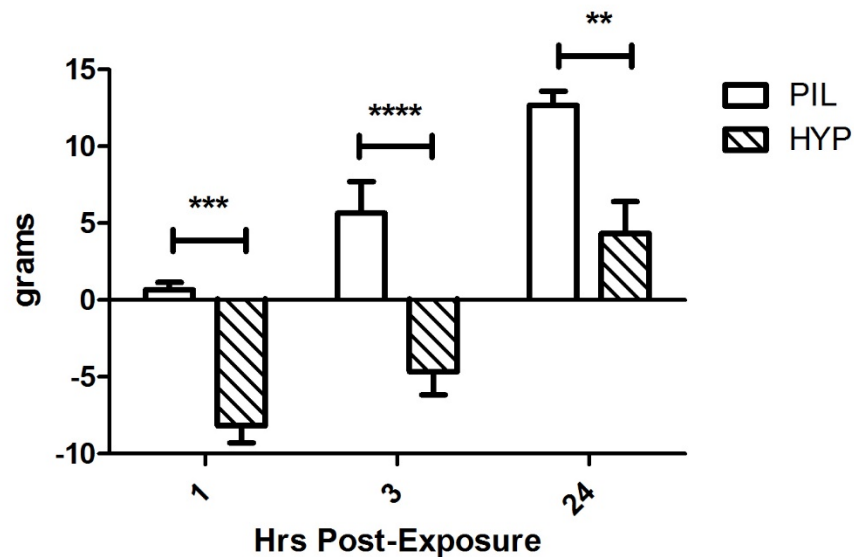


Figure 2. Comparison of Pilot- and Hypoxia-Exposed Weight Changes From Pre-Exposure to Necropsy. Differences in weight from before exposure to necropsy at 1, 3, and 24 hours post-exposure (n = 6). Data are mean \pm SEM. Two-way ANOVA and Bonferroni multiple comparisons were performed to determine significance (**p<0.01; ***p<0.001; ****p<0.0001).

4.2 HIF-1 α ELISA Results

Levels of HIF-1 α in lung tissues were below the limit of detection of the kit (data not shown). No significant differences in HIF-1 α levels were detected in brain stem, cerebellum, or cerebral cortex between CTL, PIL and HYP groups immediately after exposure (Fig. 3). Due to the very short half-life of HIF-1 α under normoxic conditions, this marker was not assayed at 1, 3, or 24 hours post-exposure.

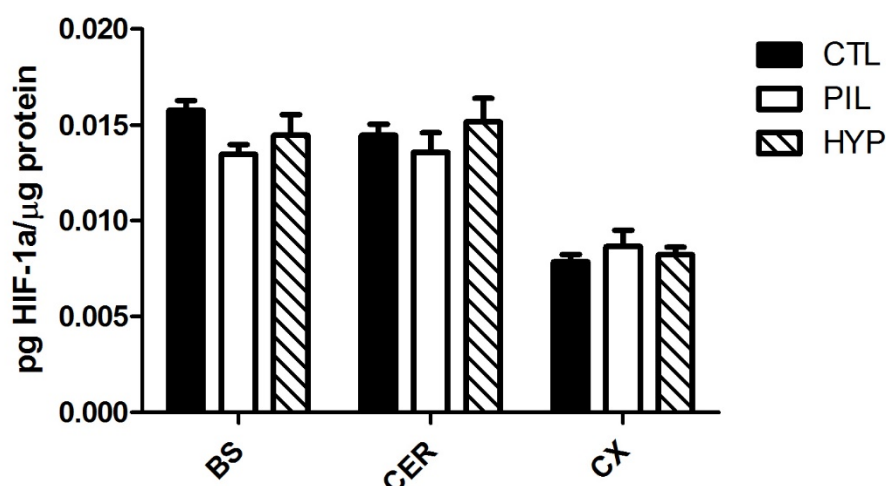


Figure 3. HIF-1 α Levels in Brain Tissue Immediately Following Exposure. Levels of HIF-1 α in three different brain regions (n = 6) immediately after Control (CTL), Pilot (PIL), or Hypoxic (HYP) exposure (BS - brain stem; CER - cerebellum; CX - cerebral cortex). Data are mean \pm standard error of the mean (SEM). Two-way Analysis of Variance (ANOVA) was performed to determine significance.

4.3 Prolyl-4-hydroxylase Alpha Polypeptide I (P4Ha1) ELISA Results

Significant decreases in P4Ha1 were observed in brain stem and cerebral cortex of HYP groups immediately following exposure ($p < 0.05$; Figures 4 and 5), and in PIL groups at 3 and 24 hours following exposure (Fig 4). Levels of the protein at other time points were not significantly different than control. P4Ha1 levels in hypoxia-exposed cerebral cortex, brain stem, and cerebellum showed a slight (but non-significant) trend upward from 0 to 24 hours post-exposure, toward control levels by 24 hours (Figs. 4, 5, and 6). There was no noticeable trend in hippocampal P4Ha1 levels, nor were there any significant differences between CTL and PIL or HYP groups in this brain region (see Fig. 7).

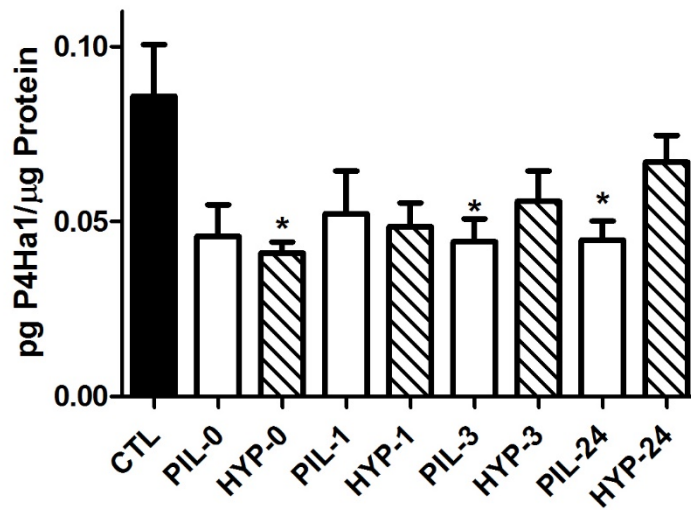


Figure 4. P4Ha1 Levels in Hypoxia- and Pilot-Exposed Brain Stem. Comparison of CTL, PIL, and HYP brain stem P4Ha1 levels at times 0, 1, 3, and 24 hours post-exposure (n = 6). Data are mean \pm SEM. One-way ANOVA and Tukey's post-test were performed to determine significance (*p<0.05). Changes noted are significantly different from CTL.

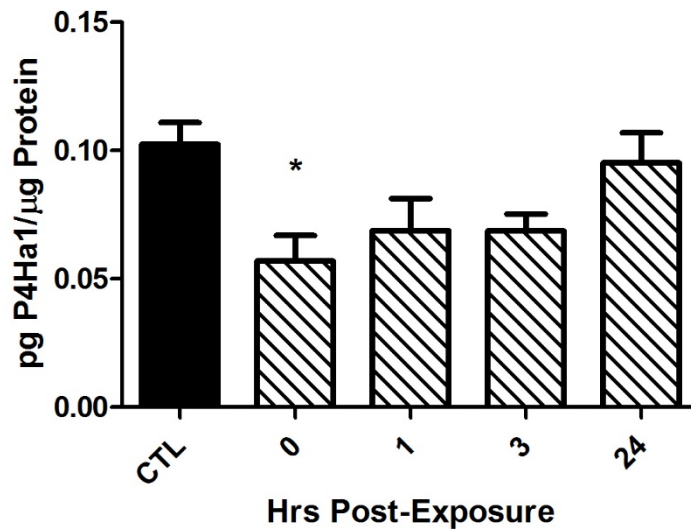


Figure 5. P4Ha1 Levels in Hypoxia-Exposed Cerebral Cortex. Comparison of HYP and CTL levels of P4Ha1 in cerebral cortex (n = 6). Data are mean \pm SEM. One-way ANOVA and Tukey's post test were performed to determine significance (*p<0.05).

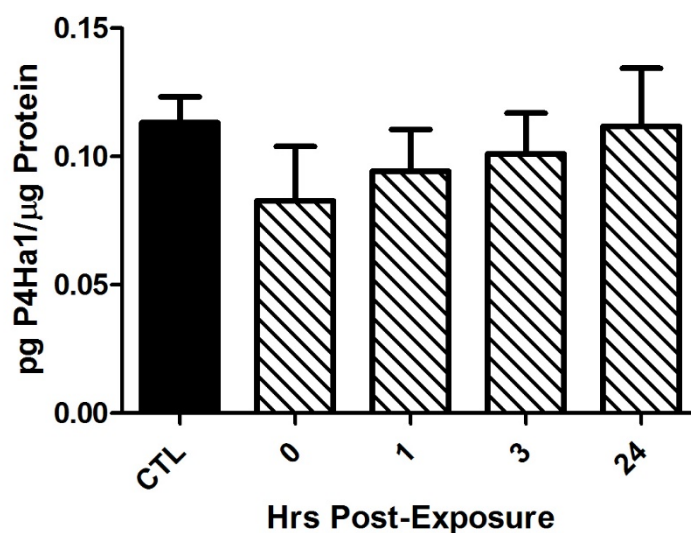


Figure 6. P4Ha1 Levels in Hypoxia-Exposed Cerebellum. Comparison of HYP and CTL levels of P4Ha1 in cerebellum (n = 6). Data are mean ± SEM. One-way ANOVA and Tukey's post test were performed to determine significance.

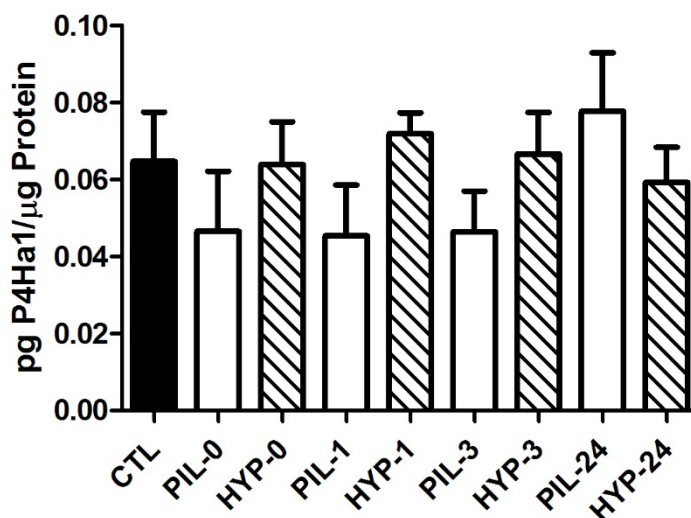


Figure 7. P4Ha1 Levels in Hypoxia- and Pilot-Exposed Hippocampus. Comparison of CTL, PIL and HYP levels of P4Ha1 in hippocampus (n = 6). Data are mean ± SEM. One-way ANOVA and Tukey's post test were performed to determine significance.

4.4 Vascular Endothelial Growth Factor ELISA Results

VEGF levels in PIL lung, brain stem, cerebellum and cerebral cortex remained essentially the same (no significant differences compared to CTL) across all time points following exposure (Fig. 8). Lung tissue VEGF levels following HYP exposure were significantly decreased at

times 1 and 3 hours post-exposure ($p < 0.05$ and $p < 0.01$, respectively). There was also a significant difference between VEGF levels at 3 hours and 24 hours following the hypoxic exposure ($p < 0.05$; Fig. 9). Of note, PIL and HYP lung VEGF levels were significantly different from each other at 0, 1, and 3 hours post-exposure ($p < 0.05$ and $p < 0.01$, and $p < 0.001$, respectively; Fig. 10). In brain stem, VEGF levels showed a different pattern, with significant increases over CTL occurring at times 0- and 24-hours post-exposure ($p < 0.05$; Fig. 11). Cerebellum and cerebral cortex did not show differential VEGF expression at any time following HYP exposure (data not shown).

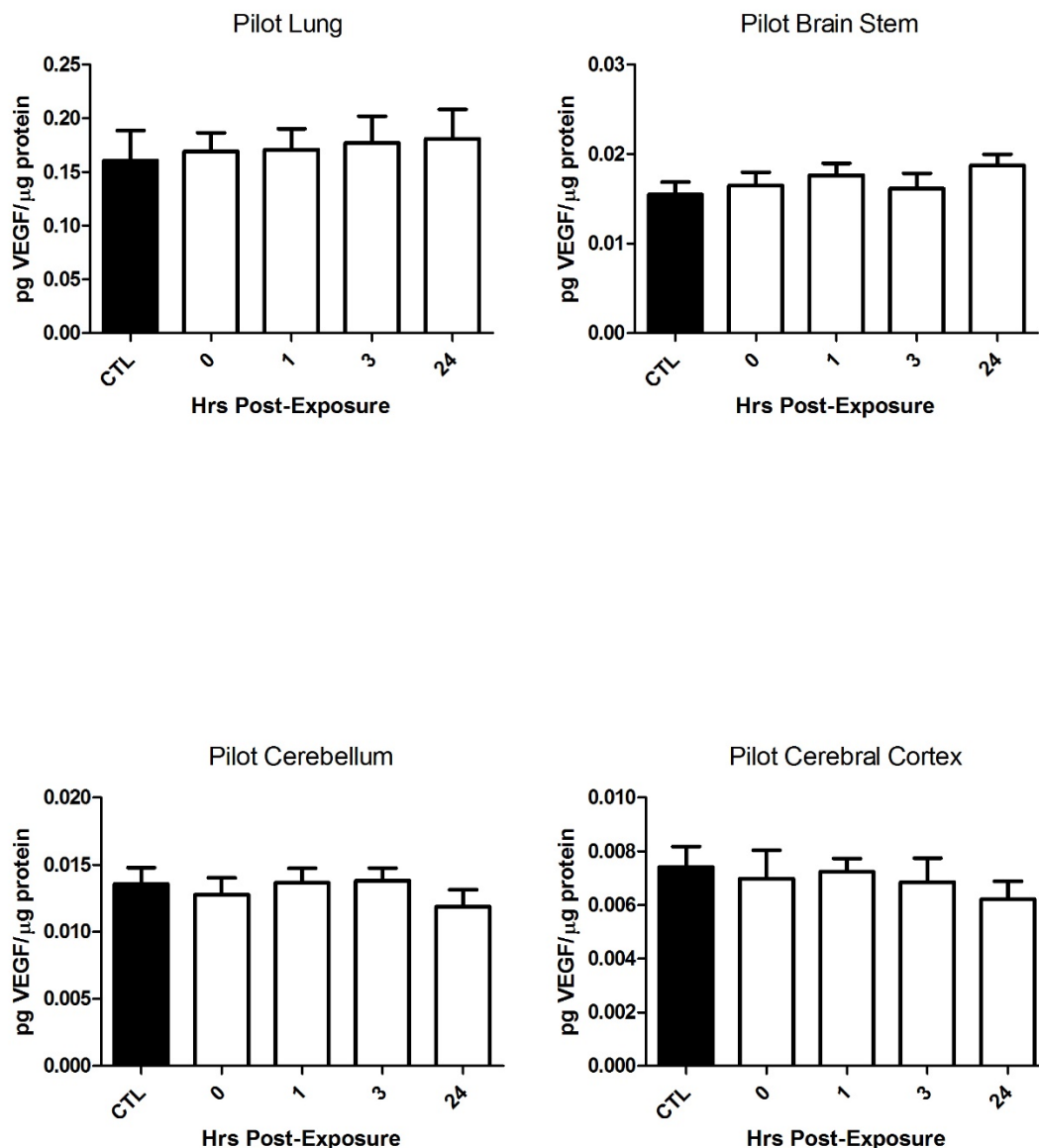


Figure 8. VEGF Levels in Lung and Brain Tissues Following Pilot Exposure. Levels of VEGF in CTL vs. PIL tissues at 0, 1, 3, and 24 hours post-exposure ($n = 6$). Data are mean \pm SEM. One-way ANOVA and Tukey's post-test were performed to determine significance.

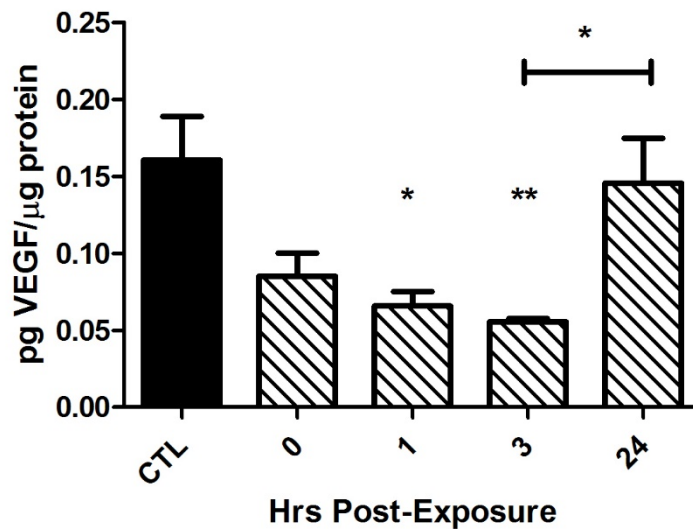


Figure 9. VEGF Levels in Lung Tissue Following Hypoxic Exposure. Levels of VEGF in CTL vs. HYP lung tissues at 0, 1, 3, and 24 hours post-exposure (n = 6). Data are mean \pm SEM. One-way ANOVA and Tukey's post-test were performed to determine significance (*p<0.05; **p<0.01).

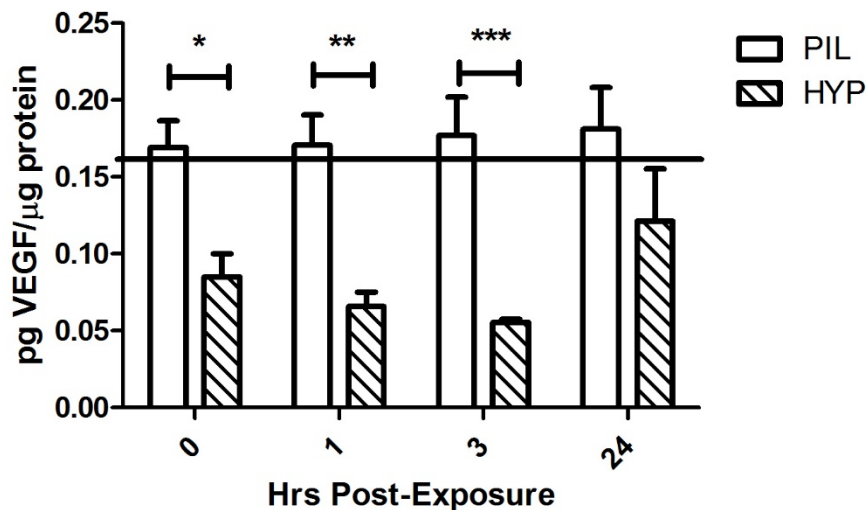


Figure 10. Comparison of VEGF Levels in Pilot and Hypoxic Lung Tissues. Levels of VEGF in PIL vs. HYP lung tissues at 0, 1, 3, and 24 hours following exposures (n = 6). Solid black line at approximately 0.16 pg represents CTL VEGF. Data are mean \pm SEM. Two-way ANOVA and Bonferroni multiple comparisons tests were performed to determine significance (*p<0.05; **p<0.01; ***p<0.001).

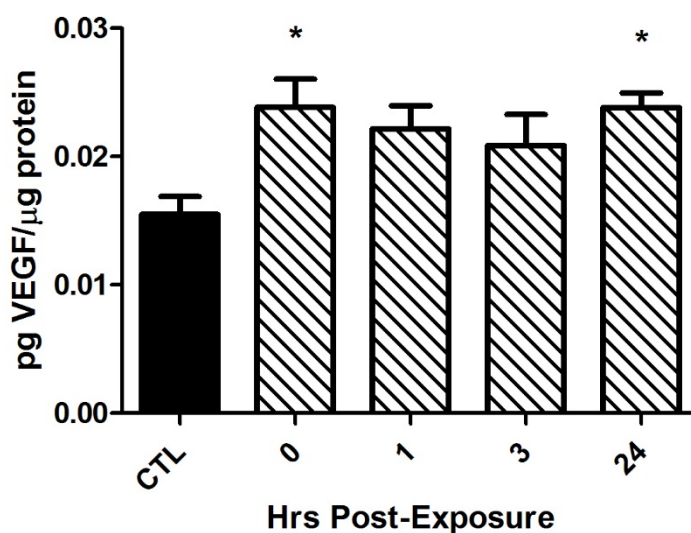


Figure 11. VEGF Levels in Brain Stem of Hypoxia-Exposed Animals. Levels of VEGF in brain stem of CTL animals compared to levels HYP animals at 0, 1, 3 and 24 hours after exposure (n = 6). Data are mean \pm SEM. One-way ANOVA and Tukey's multiple comparison test were performed to determine significance (*p<0.05).

4.5 Erythropoietin ELISA Results

Brain stem erythropoietin levels remained essentially the same as controls across all time points, regardless of PIL or HYP exposure (data not shown). In the lung, CTL and PIL EPO levels were all below the limit of detection. HYP lung EPO spiked to significantly higher than CTL values at 0, 1, and 3 hours post-exposure (p<0.01, p<0.001, and p<0.001, respectively), with the peak value measured at 1 hour post-exposure (Fig. 12). The same pattern of expression was observed in the serum of HYP animals, with significantly elevated EPO at 0, 1, and 3 hours post-exposure (p<0.01, p<0.001, and p<0.01, respectively), peaking at 1 hour post-exposure (Fig. 13). Interestingly, serum EPO levels in PIL groups were significantly decreased at 0, 1 and 3 hours post-exposure (p<0.01, p<0.001, and p<0.01, respectively), and approached CTL levels at 24 hours post-exposure (Fig. 14).

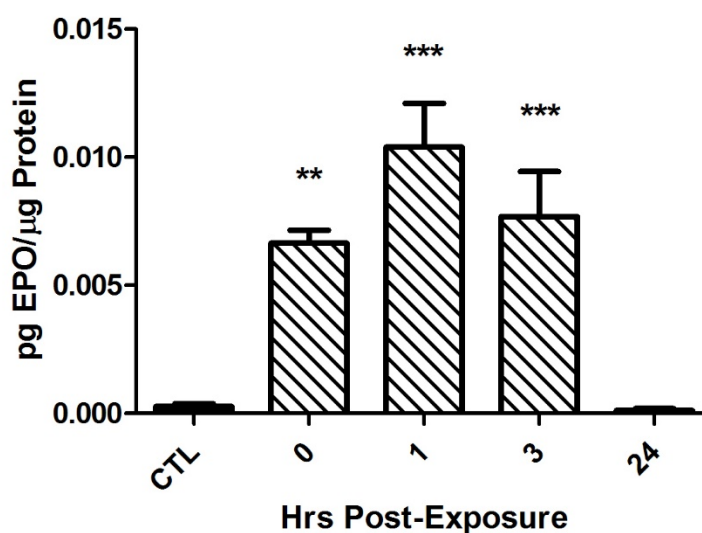


Figure 12. EPO Levels in Lung of Hypoxia-Exposed Animals. Levels of EPO in lung tissue from HYP animals and CTL animals at 0, 1, 3 and 24 hours post-exposure (n = 6). Data are mean \pm SEM. One-way ANOVA and Tukey's multiple comparison test were performed to determine significance (**p<0.01; ***p<0.001).

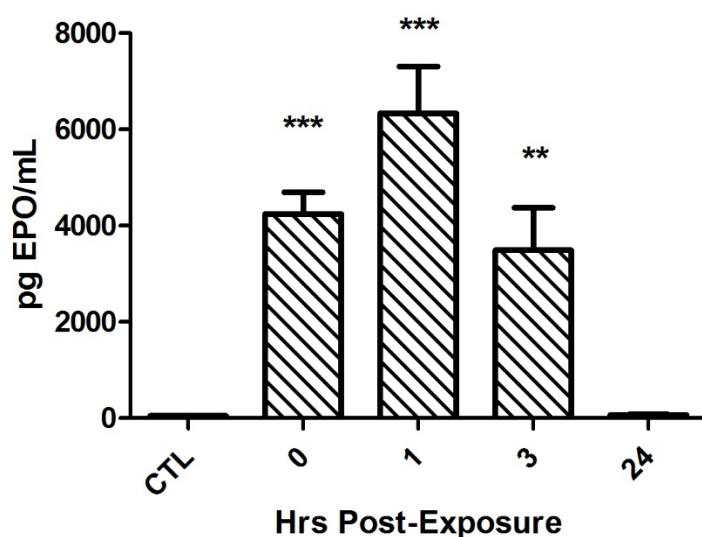


Figure 13. EPO Levels in Serum of Hypoxia-Exposed Animals. Levels of EPO in HYP vs. CTL serum at 0, 1, 3 and 24 hours post-exposure (n = 6). Data are mean \pm SEM. One-way ANOVA and Tukey's multiple comparison test were performed to determine significance (**p<0.01; ***p<0.001).

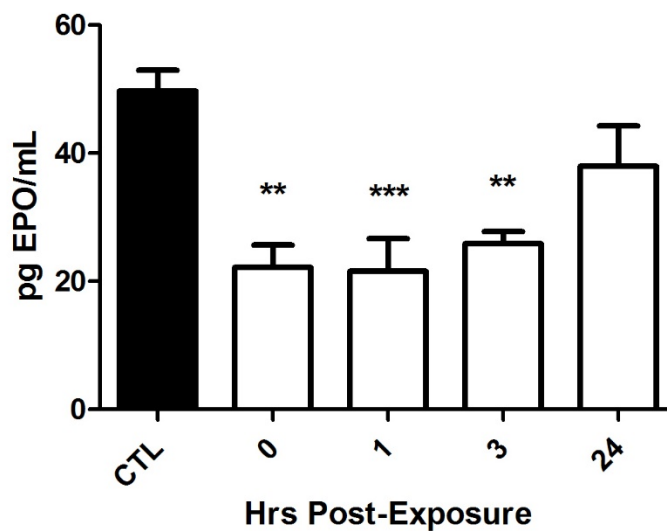


Figure 14. EPO Levels in Serum from Animals Exposed to Pilot Conditions. Levels of EPO in CTL vs. PIL serum at 0, 1, 3 and 24 hours post-exposure (n = 6). Data are mean \pm SEM. One-way ANOVA and Tukey's multiple comparison test were performed to determine significance (**p<0.01; ***p<0.001).

5.0 DISCUSSION

Humans and rodents have a two-tiered response to hypoxia. The initial response is mediated by the carotid body, which sends messages to the brain to increase respiration and heart rate to compensate for decreased oxygen in the blood. This rapid response ensures that the brain and other vital tissues continue to receive sufficient oxygen to carry out normal processes. However, when oxygen levels decrease to the point where normal processes are affected, the cellular hypoxic response is initiated. In this study, changes in body weight and four contributors to the cellular hypoxic response were examined between 0 and 24 hours after CTL, PIL and HYP exposures.

Change in body weight is often used as a measure of well-being, and body weight changes have been observed in humans upon excursion to high altitude (discussed in Rose *et al.*, 1988). While it is not clear what causes this weight loss, there are numerous potential contributors, including reduced food intake, reduced absorption of nutrients, and increased caloric expenditure during both rest and exercise (Rose *et al.*, 1988). In Rose and colleagues' study of hypobaric hypoxia, six healthy male subjects lost an average of 7.4 kg (16.3 lbs) over the course of 40 days at 240 Torr (Rose *et al.*, 1988). Weight loss was measurable within the first couple days of exposure, possibly due to anorexia from acute mountain sickness (Rose *et al.*, 1988). HYP animals in this study exhibited decreased body weights at 1, and 3, hours post-exposure, with weights increasing above pre-exposure level by 24 hours after exposure. In contrast, PIL animals showed increased body weight at all times post-exposure, indicating that they were not particularly stressed by the high altitude and high oxygen conditions (Fig. 2). The HYP rodents in this study may have experienced symptoms of acute mountain sickness (AMS), causing rapid weight loss. However,

in humans, the onset of AMS is typically between 6 and 12 hours of arrival at altitudes above 8,000 feet (Bartsch and Swenson, 2013). It is unclear how this onset scales to rodents. It seems possible that the rats may experience more rapid onset AMS than humans because of their much smaller body weight and higher rate of metabolism. The weight gain observed in PIL animals may have resulted from edema. It is not uncommon for animals to gain up to 6.8 g/day under normal housing conditions (unpublished data). The average weight gain observed in PIL animals by 24 hours after the end of the exposure (12.7 g) was nearly double that observed under normal conditions.

The half-life of HIF-1 α under normoxic conditions is under 5 min. In light of this very short half-life, it's not surprising that we did not detect elevated levels of HIF-1 α in the hypoxia-exposed animals in this study (Fig. 3). As soon as the animals are removed from the chamber and exposed to normal levels of oxygen and atmospheric pressure, HIF-1 α rapidly degrades. By the time the tissues were collected (approximately 10 min. after removal from the altitude chamber), any trace of elevated HIF-1 α was probably removed. Because of this rapid turnover, three other targets, which are detectable for longer periods of time after exposure, were also selected for analysis. The first of these is Prolyl-4-hydroxylase Alpha Polypeptide I (P4Ha1), one of the four subunits of the prolyl-4-hydroxylase 2 (PHD2) enzyme, which regulates the degradation of HIF-1 α under normoxic conditions. The other two markers are proteins that lie downstream in the hypoxic response pathway: vascular endothelial growth factor (VEGF), and erythropoietin (EPO).

Contrary to our expectations based on the literature, we did not observe any significant increases in P4Ha1 protein following hypoxia. In fact, the changes we observed in P4Ha1 levels included significant decreases in HYP brain stem and cerebral cortex immediately after exposure, and in PIL brain stem at 3 and 24 hours post-exposure (Figs. 4 and 5). Also, while not statistically significant, there was an observable trend of slightly decreased to approaching CTL values of P4Ha1 from 0 to 24 hours post-exposure in HYP cerebellum (Fig. 6). These data do not necessarily contradict the findings of Berra et al. (2003), who observed increased PHD2 levels during a hypoxic insult in isolated human cell lines. It is possible that PHD2/P4Ha1 becomes slightly depleted after a hypoxic insult, and there is a recovery period during which levels rebound to normal. It is also plausible that differences in PHD1 and PHD3 (of which P4Ha1 is also a component) expression are confounding the results, preventing direct measurement of PHD2 levels using the surrogate P4Ha1 protein. Regardless, the changes observed in P4Ha1 are neither significant nor strongly indicative of the hypoxic response.

In our animal model, VEGF levels in the lung were decreased significantly compared to CTL at 1 and 3 hours after HYP exposure (Fig. 9). This is in contrast to the anticipated rise in VEGF. By 24 hours after HYP exposure, lung VEGF levels rose to near CTL (significantly higher than the level at 3 hours post-exposure). Results from brain stem contrast with what was observed in the lungs: VEGF levels were significantly elevated over CTL at 0 and 24 hours after HYP exposure. No significant increases in VEGF were observed in the other brain regions. The brain stem is the most primitive portion of the brain and is responsible for maintaining homeostasis of the organism. It makes sense that this region of the brain would respond differently to hypoxia than other brain regions. Due to its role in responding to changing oxygen levels by signaling heart and breathing rate changes, the brain stem is the first line of defense against hypoxia. As

such, it makes sense that the brain stem would be the first to require increased vascularity, and hence would be the first place in which elevated VEGF would be observed.

Similar to the results described in the literature, we observed a distinct increase in serum and lung EPO levels immediately following hypobaric hypoxia, peaking at 1 hour after exposure and returning to CTL levels by 24 hours after the exposure (Fig. 12 and 13.) Animals exposed to PIL conditions exhibited decreased serum EPO levels at 0, 1, and 3 hours post-exposure, with levels returning to near CTL by 24 hours after exposure (Fig. 14). In all cases, EPO levels either peaked or reached their lowest points at 1 hour after exposure. These results indicate that post-exposure (or post-flight) monitoring would be optimally performed within 1 hour post-flight. Since there is usually a delay in collecting blood after a pilot lands, this biomarker would ensure the greatest likelihood of success for monitoring potential in-flight incidents. If the EPO level is depressed in the pilot it could indicate that oxygen levels were still high and an incident may not involve hypoxia. If the EPO level is elevated in the pilot, it could indicate hypoxia occurred during an incident.

Our modeling approach, described in Section 3.6 (Robinson *et al.*, 2017), allows us to compare our observed increase in VEGF due to hypoxia with changes reported in the literature. Figure 15, below, shows a comparison of the data of Schoch *et al.* (2002) (solid lines) with our measured VEGF values from Figure 11 above (green datapoint). Note that the change in VEGF over control values (about 1.4X higher) is lower than the changes observed by Schoch *et al.*; this may be at least partially accounted for by the much shorter hypoxia exposure times in our studies (4 h versus 24 h), or by species differences in metabolism and/or mRNA response between rat and mouse.

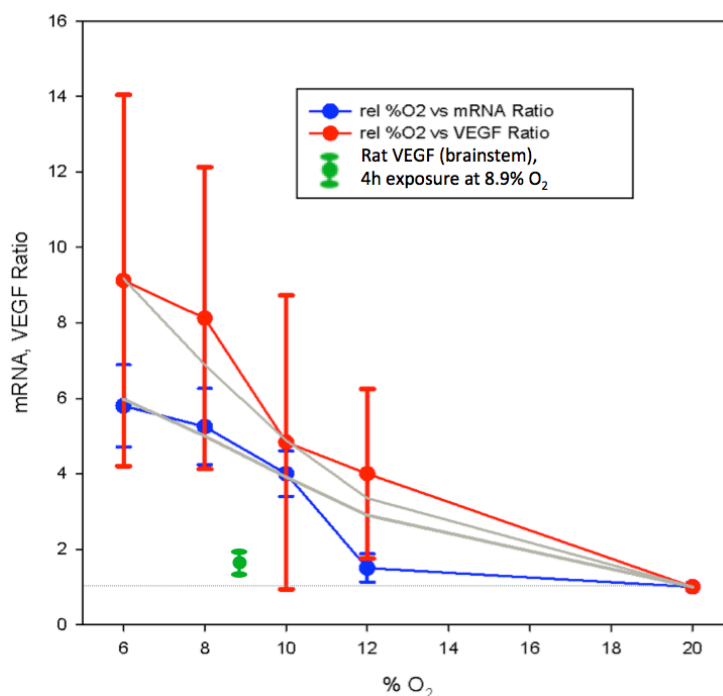


Figure 15. Transcription Signal Fits to Mouse mRNA, VEGF Data of Schoch *et al.*, 2002 Compared with Current Study. The Y axis shows the ratio of protein and message at 12 percent, 10 percent, 8 percent and 6 percent O₂, relative to normoxia (20 percent O₂), (for 24 hours) against the respective O₂ concentrations. Red curve - measured VEGF protein levels; blue curve - mRNA levels for VEGF; Gray lines - model predictions for different values of prolyl hydroxylase activity (PHD) optimized to fit each data set. Error bars represent standard deviations for the ratios. The rat VEGF protein ratio for the current study (HYP:CTL brainstem – see Figure 11 above) from 4h exposure at 22,500 feet (8.9% O₂ equivalent) is shown in green.

This study attempted to create a picture of what occurs after an acute hypobaric hypoxia exposure in a rodent model. However, the parameters, HIF-1 α , P4Ha1, and VEGF do not appear to be optimal in this regard. It is worth mentioning that each of the markers examined in this study (with the exception of VEGF in brain stem) returned to control levels by 24 hours after exposure, indicating that the first several hours following flight is a critical window for monitoring the hypoxic response. This critical window could be narrowed by additional time course studies to determine the latest possible time at which blood should be collected in order to obtain useful data. The most apparent changes occurred in serum EPO levels. It is of potential interest to see if similar elevation of serum EPO occurs in humans when exposed to shorter duration hypobaric hypoxia events, such as may occur in fighter jet cockpits. Serum collection post-flight is, theoretically, accessible and could be a useful tool for correlating in-flight physiological incidents with hypoxic conditions. Based on the results of this study, blood collection from pilots within 1 hour of landing could provide valuable information on whether or not hypoxic conditions were encountered. Additional work should be performed to determine the optimal times post-flight for blood collection in order to gain a broader understanding of what occurs in the cockpit.

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LIST OF ACRONYMS

AMS	Acute mountain sickness
ANOVA	Analysis of variance
AVMA	American Veterinary Medical Association
BCA	Bicinchonic acid
BS	Brain stem
CER	Cerebellum
CTL	Control
CX	Cerebral cortex
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
G-LOC	G-related loss of consciousness
HIF-1 α	Hypoxia-inducible factor-1 α
HRE	Hypoxia responsive element
HYP	Hypoxic
IACUC	Institutional Animal Care and Use Committee
NRC	National Research Council
OBOGS	On-board oxygen generating system
P4Ha1	Prolyl-4-hydroxylase Alpha Polypeptide I
PHD2	Prolyl-4-hydroxylase 2
PIL	Pilot control
SEM	Standard error of the mean
SS	Steady-state
VEGF	Vascular endothelial growth factor